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CASE REPORT

SEROLOGICAL STUDY OF AN ALLERGIC AGRANULOCYTOSIS DUE TO NORAMIDOPYRINE

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SUMMARY

An allergic agranulocytosis induced by amidopyrine and triggered by noramidopyrine was studied. Leucoagglutinating and leucocytotoxic antibody, active only in the presence of the drug, was demonstrated. The antibody was stable, giving a titre from 1·34 to 1·62 and was present in the IgM (19S globulin) and in the IgG (7S globulin) serum fractions. The site of drug fixation was studied by use of iodo-antipyrine labelled with ^{131}I; a stable fixation was demonstrated on to the IgM and IgG globulins. Special emphasis is given to cross-reaction with compounds related to amidopyrine.

INTRODUCTION

Agranulocytosis due to amidopyrine has been described by: Madison & Squier (1934), Dameshek & Colmes (1936), Müller (1954), Bernard, Najean & Binet (1956), Dyrbye & Huidberg (1961) and Thygessen (1962). Agranulocytosis due to noramidopyrine has been described by Kalicinsky & Pasko (1957), Kowalczyk & Urasinski (1957) and Huguley (1964). Cases have been published in which the existence of a leucoagglutinin was demonstrated during the agranulocytic crisis (Moeschlin & Wagner, 1952; Moeschlin & Moreno, 1954; Miescher, Straessle & Miescher, 1955; Dausset, 1956a). The leucoagglutinin was active without addition of the sensitizing drug, as an amount of drug, sufficient to induce the reaction, was probably still present in the patient's body. The activity of the leucoagglutinin could not be demonstrated by addition of the drug to the patient's serum after recovery (Moeschlin & Wagner, 1952). Magis *et al.* (1962) published the first case in which a stable antibody was shown to be active only after addition of amidopyrine to the patient's serum.

The case we present here is a new one of an allergic agranulocytosis, induced by amidopyrine and triggered by noramidopyrine with presence of a stable antibody. The antibody was detected during the disease which lasted 15 days. The titre of the antibody remained stable for a year after complete clinical recovery. This antibody cross-reacted with a certain

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number of compounds having structures closely related to that of amidopyrine. In an attempt to elucidate the immunoallergic mechanism of the reaction a radioactive labelled drug was used. As amidopyrine was not available in labelled form iodoantipyrine labelled ^{131}I was employed. This compound, closely related to amidopyrine and noramidopyrine, is capable also of inducing leucoagglutination.

Case Report

Mrs Gr. is 35 years old, with no history of illness. Since 1957 she has suffered from arthritic rheumatism for which she took no drug other than acetyl-salicylic acid. In October and November 1964 she twice took 0·50 g of amidopyrine for painful menstruation. From 15 to 26 January 1965, she took noramidopyrine for another arthritic attack, the dose being: 250 mg, four times a day, the

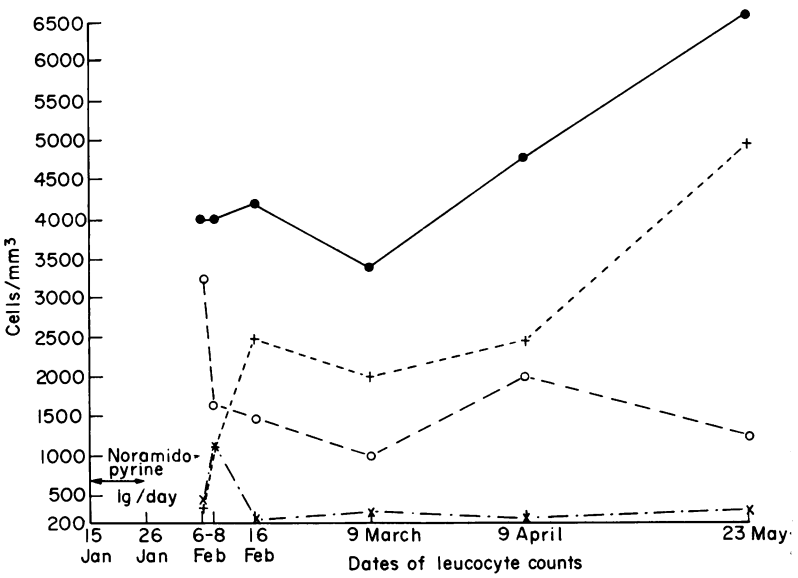


FIG. 1. Evolution of the absolute white blood cell count. ●, Leucocytes; ○, lymphocytes; +, polymorphonuclear neutrophils; ×, monocytes.

total dose was 11 g. On 28 January she developed tonsilitis, followed 6 February by a necrotizing angina with lymphadenopathy and high fever. On 6 February a leucocyte count showed 4000 WBC/mm³ and the differential count was: polymorphonuclear neutrophils, 7%; lymphocytes 79%; monocytes, 14%. Penicillin, rovamycin and pancyclin were administered. On 8 February the count showed 4000 WBC/mm³ and the differential count was: polymorphonuclear neutrophils, 27%; lymphocytes, 43% and monocytes, 30%. On 9 February the leucoagglutination test carried out in the presence of amidopyrine and of noramidopyrine was positive. On 16 February the count had almost returned to normal, 4200 leucocytes/mm³, with 60% polymorphonuclear neutrophils, 35% lymphocytes and 5% monocytes (Fig. 1). However, the same day a marrow examination showed an increase in the young forms with no maturation arrest. The differential count was myeloblasts, 5%; promyelocytes, 4%; myelocytes, 6%; metamyelocytes, 21%; segmented neutrophils, 30%; basophilic neutroblasts, 2%; polychromatophilic erythroblasts, 8%; acidophilic erythroblasts, 17% and lymphoid cells, 7%. The megakaryocytes were normal. Throughout the evolution of the disease there was neither anaemia nor thrombopenia, and recovery was complete.

METHODS

Serology

Three serological techniques were used.

(a) *The leucoagglutination technique* using the method described by Dausset (1956b) and modified by Engelfriet & van Loghem (1961).

(b) The *lymphocytotoxicity and granulocytotoxicity test.* The lymphocytes were separated by sedimentation from leucocyte suspensions which had been obtained from defibrinated blood (Dausset, 1956b) using the method of Thierfelder (1964). The granulocytes were obtained from the leucocyte suspension by the method of Wildy & Riedley (1958). The leucocytotoxicity test was carried out following the technique of Engelfriet & Eijsvogel (1965) using rabbit serum instead of human serum as source of complement.

For the various tests we used 0·05 ml of the patient's serum, 0·05 ml of the drug solution and 0·05 ml of leucocyte, lymphocyte or granulocyte suspension. Controls were carried out simultaneously in all the tests using a normal serum from a non-transfused AB group male donor, the patient's serum in the absence of the drug, and the drug solution alone.

(c) *The complement fixation test on leucocytes.* This test was performed according to the technique of Kabat & Mayer (1961).

Serum fractionation

The IgG (7S γ-globulins) proteins were obtained by chromatography of the serum on DEAE-cellulose using a modification of Levy & Sober's technique (1960), 2 ml of the pathological serum and 2 ml of a pool of normal sera were, respectively, fractionated on a 1×25 cm column of DEAE-celluose (2 g of DEAE-cellulose for 100 mg protein) equilibrated with a 0·05 M, pH 8·1, phosphate buffer. The elution was made with the same buffer, the flow of the column being 20 ml/hr. The first protein fraction eluted was concentrated by dialysis against polyethylene-glycol. It contained mainly IgG globulins as confirmed by micro-immunoelectrophoresis using the method of Sheidegger (1955) with an LKB apparatus.

The IgM (19S γ-globulins) proteins were obtained by gel filtration on Sephadex G-200 according to the method of Flodin & Killander (1962). Five millilitres of the pathological serum and 5 ml of a pool of normal sera were respectively fractionated on a 3×100 cm Sephadex G-200 column, equilibrated with a pH 8·0 buffer (0·1 M-Tris–HCl + 1 M-NaCl). The elution was made with the same buffer. The flow of the column was 5 ml/hr. The IgM proteins were collected after the passage of a volume of eluate equal to the void volume of the column. This fraction was concentrated by dialysis against polyethylene glycol. The identity of the protein was confirmed by micro-immunoelectrophoresis. The fraction contained IgM globulins with some β-lipoproteins. The protein concentration was followed during the chromatography on DEAE-cellulose or during the gel filtration on Sephadex by absorption at 280 mμ using a Beckman DU spectrophotometer.

The protein concentration of the sera and of the isolated protein fractions were estimated using Lowry's technique (Lowry *et al.*, 1951).

Treatment of the immunoglobulins with 2-mercaptoethanol

Both the pathological serum and the pool of normal sera were treated with 2-mercapto-

ethanol at a final concentration of 0·2 M, incubated for 1 hr at laboratory temperature, then dialysed for 24 hr against pH 7·2 buffered saline (Grubb & Swahn, 1958).

Test with the ^{131}I-labelled iodoantipyrine

To 1 ml of the patient's serum containing 60 mg proteins, was added 1 ml of a solution containing 44 μg of ^{131}I-labelled iodoantipyrine, with a specific radioactivity of 100 μC giving a count of 8×10^6 counts/min. (The iodoantipyrine used for these experiments was Codex grade purity and did not contain non-iodinated iodoantipyrine. The precise place of the iodine atom in the iodoantipyrine molecule is not known; iodoantipyrine is considered as an iodide of antipyrine presenting tautomeric structures. Nevertheless, considering the conditions of the iodoantipyrine synthesis, the iodine atom cannot be located on the phenyl ring, but must be located on the pyrazole ring.)

An equal amount of the iodoantipyrine solution is added to 1 ml of the pooled normal sera (65 mg proteins) as control.

After 1 hr incubation at 37°C, the two samples were dialysed at +4°C against 200 volumes of pH 7·2 buffered saline, changed four times daily, during 48 hr. The residual radioactivity was measured with a scintillation counter (low background scintillation counter Tracerlab Waltham, Massachusetts, U.S.A.).

Other samples of the pathological serum and of the normal pooled sera, similarly treated with ^{131}I-labelled iodoantipyrine, were submitted to gel filtration through a Sephadex G-200 column according to the technique of Killander (1963). The filtration was performed on a 3×100 cm Sephadex G-200 column equilibrated with a pH 8·0 buffer (0·1 M-Tris–HCl + 1 M-NaCl). The elution was carried out with the same buffer, the flow of the column being 5 ml/hr and the eluate collected in fractions of 5 ml. The protein concentration was followed during the filtration by absorption at 280 mμ on a Beckman DU spectrophotometer, and the radioactivity followed on each fraction with a scintillation counter. The four protein fractions obtained, according to Killander & Hogman (1963), were identified by micro-immunoelectrophoresis. The IgM proteins and the β-lipoproteins were found in the first 120 ml of eluate, the IgA proteins in the 80 ml which followed, the IgG proteins between 200 and 280 ml of eluate and finally in the last 350 ml of eluate albumin predominated with α_1- and β_2-globulins as major contaminants.

RESULTS

Leucoagglutination

The titres of the leucoagglutinin, as tested with the patient's leucocytes and with leucocytes of healthy individuals, varied from 1:32 to 1:128 in the presence of amidopyrine and noramidopyrine. Table 1 shows that the antibody persisted in the patient's serum, its titre being practically unchanged 4 months after the onset of the disease. It was still present at the same titre after 1 year.

(a) *Leucoagglutination by the immunoglobulin fractions.* The isolated IgM fraction which contained 1 mg protein/ml, was active at a dilution of 1:4. The isolated IgG fraction containing 10 mg/protein ml was active at a dilution of 1:8 to 1:16. For both fractions the activity could only be detected in presence of amidopyrine or noramidopyrine. The patient's serum treated with 2-mercaptoethanol gave positive leucoagglutination at a dilution of 1:4 as compared to the dilution of 1:32 of the untreated serum.

(b) *Leucoagglutination in the presence of drugs related to amidopyrine and noramidopyrine.* Table 2 and Fig. 2 show compounds related to amidopyrine. All of them are 1-phenyl-pyrazole-5-one derivatives. They include amidopyrines metabolites, e.g. antipyrine,4-amino-antipyrine and acetyl-antipyrine. Molecular associations of amidopyrine with different

TABLE 1. Evolution of the antibody titre in the course of time

Dates of the samples	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
9 February 1965	+++	+++	+++	+++	+++	+++	++	+
15 March 1965	+++	+++	+++	+++	++	+	−	−
5th April 1965	+++	+++	+++	+++	++	+	(+)	−
18th May 1965	+++	+++	+++	+++	++	+	−	−
5th June 1965	+++	+++	+++	+++	+++	++	+	(+)
23rd April 1966	+++	+++	+++	+++	+++	++	(+)	−

Test carried out on the same day with the same leucocytes the sera were stored at −20°C.

compounds such as barbiturates, choral and urethane have also been tested. With these molecular associations amidopyrine behaves as if isolated; these compounds are not listed in Table 2.

All compounds in Table 2 bear identical constituents in —3 and —5 on the pyrazole ring,

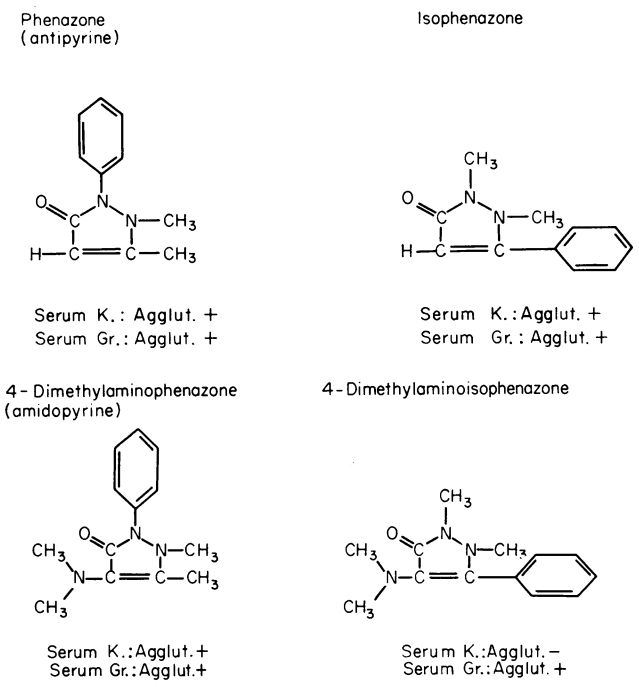


FIG. 2. Spatial structures of antipyrine and amidopyrine and of their isomers with their capacity to cause agglutination.

methyl group $-CH_3$ in —3 and an oxygen atom $=0$ in —5. Two compounds, 1-phenyl-pyrazole-3-one and 1-phenyl, 5-methyl-pyrazole-3-one gave also positive leucoagglutination.

Table 3 shows a list of 1-phenyl-pyrazole derivatives which differ from 1-phenyl-pyrazole-5-one derivatives in that the oxygen atom $=0$ in —5 has been replaced. These compounds gave also positive leucoagglutinations.

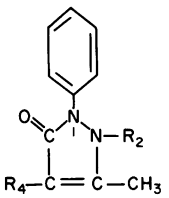


TABLE 2. Compounds, belonging to the phenazone group, which induced leucoagglutination when added to the patient's serum

No.	Formula	R_2	R_4
1	Phenazone (antipyrine)*	$-CH_3$	$-H$
2	4-Amio-antipyrine*	$-CH_3$	$-NH_2$
3	4-Acetyl-amino-antipyrine*	$-CH_3$	$-NH-COCH_3$
4	4-Formyl-antipyrine*	$-CH_3$	$-C(=O)-H$
5	4-Propylphenazone*	$-CH_3$	$-CH(CH_3)_2$
6	Homophenazone*	$-C_2H_5$	$-H$
7	4-Dimethylaminophenazone (amidopyrine)*	$-CH_3$	$-N(CH_3)_2$
8†	4 — Diethylaminophenazone*	$-CH_3$	$-N(C_2H_5)_2$
9	Noramidopyrine*	$-CH_3$	$-NH-CH_3$
10	Noramidopyrine methanesulphonate	$-CH_3$	$-N(CH_3)-CH_2-SO_3H$
11	Melaminsulphone*	$-CH_3$	$-NH-CH_2-SO_3H$
12	4-Methyl-phenazone*	$-CH_3$	$-CH_3$

* Derivatives studied against Gr.'s and K.'s antibodies (Magis *et al.*, 1962; Thierfelder *et al.*, 1964).

† Derivative No. 8 gave negative reaction with K.'s antibody.

Table 4 includes derivatives of 1-phenyl-pyrazole-5-one differing by the addition of chlorine atoms —Cl or an amino group $-NH_2$ on the phenyl ring. The leucoagglutination reactions were either weakly positive or negative according to the position of —Cl on the phenyl ring; weakly positive when the addition was in position —2', negative when in —3' or

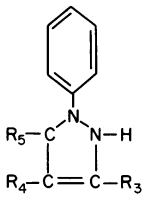


TABLE 3. 1-Phenyl-pyrazole derivatives which induced leucoagglutination when added to the patient's serum

No.	Formula	R_1	R_3	R_4	R_5
13	1-Phenyl, 4-aminopyrazole*		-H	$-NH_2$	-H
14	1-Phenyl, 4-dimethylamino-pyrazole*		-H		-H
15	1-Phenyl, 3, 5-dimethyl-4-dimethylaminopyrazole*		$-CH_3$		$-CH_3$
16	1-Phenyl, 3-methyl-4-dimethylaminopyrazole		$-CH_3$		-H
17	1-Phenyl, 5-dimethyl-aminopyrazole*		-H	-H	
18†	1-(2',6'dimethyl) Phenyl, 4-dimethylaminopyrazole*		-H		-H
19†	1-Phenyl, 4-dimethylamino-5-methylpyrazole*		-H		$-CH_3$

* Derivatives studied against Gr.'s and K.'s antibodies (Magis *et al.*, 1962; Thierfelder *et al.*, 1964).

† Derivatives Nos. 18 and 19 gave negative reactions with K.'s antibody.

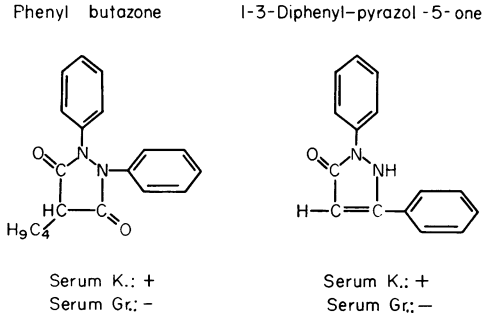


FIG. 3. Spatial structures of diphenyl-pyrazolone derivatives with their capacity to cause agglutination.

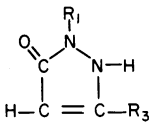
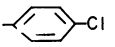
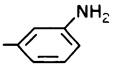
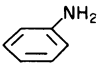


TABLE 4. 1-Phenyl-pyrazole-5-one derivatives which did not induce leucoagglutination when added to the patient's serum

No.	Formula	R_1	R_3
20	1-(3'chloro phenyl) 3-Methyl-pyrazole-5-one*		$-CH_3$
21	1-(4'chloro phenyl)3-Methyl-pyrazole-5-one*		$-CH_3$
22†	1-(2'chloro phenyl)3-Methyl-pyrazole-5-one*		$-CH_3$
23	1-(3'amino phenyl) 3-Methyl-pyrazole-5-one*		$-CH_3$
24	1-(3'amino phenyl)3-Carboxy-pyrazole-5-one*		-COOH

* Derivatives studied against Gr.'s and K.'s antibodies (Magis *et al.*, 1962; Thierfelder *et al.*, 1964).

† Derivative No. 22 induced only a weak positive reaction. None with K.'s antibody, all others gave negative reactions with both sera.

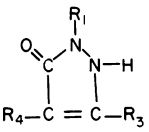


TABLE 5. Pyrazole-5-one derivatives which induced only weak or no leucoagglutination when added to the patient's serum

No.	Formula	R_1	R_3	R_4
25†	4-Amino-pyrazole-5-one*	-H	-H	$-NH_2$
26†	3-Amino-pyrazole-5-one*	-H	$-NH_2$	-H
27‡	1-Amino-pyrazole-5-one*	$-NH_2$	-H	-H
28‡	3-Methyl-pyrazole-5-one*	-H	$-CH_3$	-H

* Derivatives studied against Gr.'s and K.'s antibodies (Magis *et al.*, Thierfelder *et al.*, 1964).

† Derivatives Nos. 25 and 26 induced only weak positive reactions.

‡ Derivatives Nos. 27 and 28 did not cause agglutination.

—4′. The same must be said when the addition on the phenyl ring was the group —NH_2.

Fig. 3 shows the diphenyl-pyrazole-5-one derivatives with which the leucoagglutination is positive with the serum Gr.

Table 5 shows a list of pyrazole-5-one compounds without a phenyl ring in position —1

TABLE 6. Lymphocytotoxicity and granulocytotoxicity (% of the cells stained with trypan blue)

		Dilution of Gr.'s serum						
		1:1	1:2	1:4	1:8	1:16	1:32	1:64
Patient's cells	Lymphocytes+ granulocytes	82*	64	64	58	26	17	5
	Lymphocytes*	7	49	43	44	25	14	5
Homologous cells	Lymphocytes+ granulocytes	69	55	26	18	17	5	—
	Lymphocytes*	42	37	30	18	13	6	—
	Granulocytes*	55	51	32	18	6	5	—

* The test is considered as positive if more than 16% of cells take up the dye.

on the pyrazole ring. These compounds gave negative or very weak positive leucoagglutination reactions.

Lymphocytotoxicity and granulocytotoxicity

The antibody, in the presence of amidopyrine or noramidopyrine, was active against the patient's leucocytes and against control leucocytes. It was also active against the patient's

TABLE 7. Fixation of iodoantipyrine labelled with ^{131}I on serum proteins

	Activity (counts/min)	Fixed iodoantipyrine (μg)
Patient's serum after dialysis (1 ml)	1,363,000	7·48
Pool of normal sera after dialysis (1 ml)	312,000	1·77

lymphocytes and control lymphocytes. It reacted to the same titres with the patient's granulocytes and control granulocytes (Table 6).

^{131}I-labelled iodoantipyrine

The patient's serum was treated with ^{131}I-labelled iodoantipyrine, and dialysed, and agglutinated homologous leucocytes without further addition of the drug. Estimated from the residual radioactivity counts/min the patient's serum retained 7·48 μg (1,353,000 counts/min) of iodoantipyrine after dialysis, whereas the pooled control sera retained only 1·77 μg (312,000 counts/min) as shown in Table 7.

Gel filtration of the serum labelled with [^{131}I]iodoantipyrine

In the gel filtration on Sephadex G-200 column of the patient's serum labelled with [^{131}I]-iodoantipyrine, the radioactivity showed three maxima. The first appeared during the elution of the IgM proteins, the second during the elution of the IgA proteins, and the third during the elution of the IgG together with the proteins which were eluted immediately afterwards, e.g. the β_2- and α_1-globulins and the albumin. A pool of normal sera was used as

TABLE 8. Fixation of iodoantipyrine labelled with ^{131}I on immunoglobulin fractions

Immunoglobulin fractions	Patient's serum* (counts/min activity)	Pool of sera* (counts/min activity)
IgM	797	0
IgA	1368	0
IgG	5079	1978
β_2- and α_1-globulins and albumin	1398	5597

* The gel filtration of the patient's serum was made about 2 weeks after that of the pool of normal sera. The figures of the counts/min expressing the activity of each fraction have not been adjusted to take into account the loss of activity of the ^{131}I.

control. It was treated in the same manner as described above for the patient's serum. A single peak of radioactivity was observed which coincided with the elution of the IgG, β_2, α_1-globulins and albumin (Table 8).

Complement fixation test

The maximum amount of complement which could be fixed by the patient's serum was 8–10 units, and this only in the presence of amidopyrine and the patient's leucocytes or foreign leucocytes.

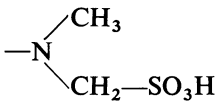
DISCUSSION

From the patient's clinical history it appears very likely that she has been sensitized by amidopyrine, and that the re-immunization was due to noramidopyrine. Both compounds have the same basic structure 1-phenyl-pyrazole-5-one, but differ in the nature of the substituents in —R_4. Cross-reactions have been obtained in leucoagglutination with other derivatives of 1-phenyl-pyrazole-5-one.

From this study we observed that a single modification on the basic structure of amidopyrine's formula could, in some cases, induce differences in the leucoagglutination reactions. Thus modification of the —R_1 substituent group, by adding a —Cl atom in position —2′ (No. 22, Table 4) produced weak positive reactions, whereas the addition of a —Cl atom in —3′ or —4′ (Nos. 20 and 21 of Table 4) or of an amino group —NH_2 in position —3′ (Nos. 23 and 24, Table 4) resulted in negative reactions. Thus it appears that the addition of a

chlorine atom on the phenyl ring in —3′ and —4′ gives a spatial configuration to that part of the molecule that is no longer compatible with the antibody's specificity.

The different modifications in the structure of the $—R_4$ substituent group that we have tested lead to positive results, and the intensity of the leucoagglutinations obtained was equal to that observed in the presence of amidopyrine or noramidopyrine (Table 2). The leucoagglutination remained positive when the $—R_4$ substituent group was small, e.g. antipyrine (Table 2; No. 1) or large such as $—NH—CH_2—SO_3H$ (No. 11) or even as large as


(No. 10).

It thus appears that the steric hindrance which could be due to the size of the $—R_4$ substituent group does not render the reaction negative.

The $—R_5$ substituent group was modified by replacing the oxygen atom $=O$ by either a methyl group $—CH_3$ (No. 15, Table 3) or by an hydrogen atom —H (No. 16, Table 3) without inducing negative reactions.

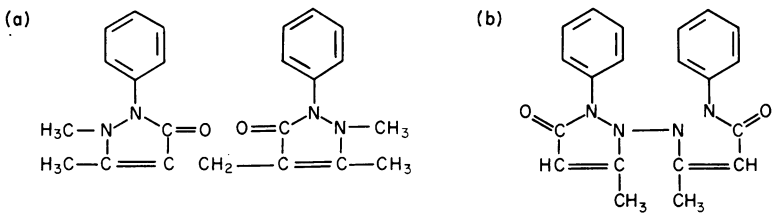


FIG. 4. Spatial structure of: (a) di-antipyrine methane, and (b) bis-pyrazolone. Neither of these compounds caused leucoagglutination with either of the two sera Gr. or K.

Two simultaneous modifications, one on the $—R_2$ and the other on the $—R_4$ substituent group ($—C_2—H_5$ for the former, —H for the latter), also gave positive reactions (No. 6, Table 2). Likewise, two other compounds tested, presenting simultaneous modifications on $—R_3$ and $—R_5$ substituent groups, gave positive reactions. They are l-phenyl, 5-methyl-pyrazole-3-one and 1-phenyl-pyrazole-3-one. As these compounds are derivatives of 1-phenyl-dyrazole-3-one they cannot figure in Table 2 in which all compounds listed are 1-phenyl-pyrazole-5-one derivatives.

Positive reactions were also observed with compounds presenting other simultaneous modifications of the $—R_3$ and $—R_5$ substituent groups (No. 14, Table 3). In this compound the $—R_4$ substituent group is also changed, but as it has been mentioned earlier, modifications of the $—R_4$ substituent group have no influence on the reaction.

On the other hand, compounds such as diantipyrine and bis-pyrazolone (Fig. 4) are not capable of inducing leucoagglutination although both are 1-phenyl-pyrazole-5-one derivatives, since these two compounds are merely symmetric doubles of antipyrine for diantipyrine methane, and of 1-phenyl, 4-methyl-pyrazole-5-one for bis-pyrazolone, respectively, without any other alteration of the single molecule. One must admit that the large spatial configuration of these two molecules presents such steric hindrance, that they are no more compatible with the antibody's specificity.

Finally, when the pyrazole ring no longer carried the phenyl substituent in position —1 (Table 5) and when it included at least two other modifications of the basic structure, the reactions were either weakly positive (Nos. 25 and 26, Table 5) or negative (Nos. 27 and 28, Table 5) even when the incubation was lengthened to 3 hr.

Thus, it appears that while the presence of the phenyl ring brings about stronger reactions, its position can vary, as shown in Fig. 2. Positive reactions were obtained when the phenyl ring was located in $—R_3$ instead of $—R_1$. This is the case for the amidopyrine isomer as well as for the antipyrine one.

We also compared the specificity of our patient's serum with that of another patient (K.) whom we have previously studied (Magis *et al.*, 1962). This serum was also an anti-amidopyrine leucoagglutinating serum. Both Gr.'s and K.'s sera were tested in parallel for leucoagglutination with thirty-three of the thirty-seven compounds having similar structures to that of amidopyrine, which we had at our disposal. These compounds are marked with an asterisk in Tables 2–5. Although most of these compounds gave the same positive or negative reactions with the two sera, some discrepancies were observed.

(a) 4-Diethylaminophenazone (No. 28, Table 2) and 4-dimethylamino-isophenazone (Fig. 2) gave negative reactions with K.'s serum. The same applied to Nos. 18 and 19 of Table 3, No. 22 of Table 4 and No. 25 of Table 5.

(b) Phenylbutazone and 1, 3-diphenyl-pyrazole-5-one (Fig. 3) which gave positive leucoagglutinations with K.'s serum did not induce leucoagglutination when added to Gr.'s serum. Yet the leucoagglutination titre of K.'s serum was weaker (1:4 to 1:8) than that of Gr.'s serum (1:32 to 1:64) in the presence of amidopyrine.

Thus, the specificity of the former is different from that of the serum of the case studied here. Indeed it seems unlikely that K., who had been sensitized against amidopyrine, was also sensitized against phenylbutazone, particularly since phenylbutazone was not yet in use at the time of the occurrence of her first episode of agranulocytosis.

So far we have been unable to draw from this comparative study any general conclusions concerning the antigenic structure of the haptene.

The leucoagglutinating antibody was also cytotoxic *in vitro* and this cytotoxicity was directed not only against the granulocytes but also against the lymphocytes. This observation was in disagreement with those made *in vivo*, the latter showing, 11 days after the discontinuation of the drug, only a decrease of the granulocyte number. A possible explanation can be given to this observation, but it cannot be verified. In the present case the apparent blocking of the marrow maturation at the promyelocyte stage could be due to a destruction of the more mature cells or to an alteration in the formation or maturation of the cells through a direct toxic effect of the drug. It is not known if a similar effect could take place in the lymph nodes. However, the difference in the length of the maturation time of granulocytes and of lymphocytes, and the difference of survival time in the peripheral blood of both type of cells (Athens *et al.*, 1961; Cronkite *et al.*, 1964), may epxlain the difference observed between the two series.

Two theories have been put forward regarding the drug fixation site. According to Ackroyd (1954, 1962) the drug binds to cellular proteins by a very loose link, the antibody acting on the antigenic complex thus formed *in vivo*. According to Miescher *et al.* (1955) and to Shulman (1963, 1964) the drug binds by means of a stable link to a macromolecule of the plasma, forming a soluble complex which reacts with the antibody.

We made an attempt to ascertain whether the drug could fix specifically to the antibody

we had at our disposal. Our observations were in favour of a specific binding of the drug to the proteins as the amount of a labelled iodoantipyrine fixed to the proteins of the patient's serum was four times higher than that fixed to the proteins of the pool of normal sera (Table 7). This binding seemed stable since in both cases, after 48 hr dialysis, against a large volume of dialysate changed several times, it was not possible to remove all the drug. Ackroyd (1962) had shown that the linkage of the Sedormid to the proteins of a sensitive patient was labile in different conditions. After addition of Sedormid to the serum of a Sedormid sensitive patient, he precipitated the globulins by ammonium sulphate. The precipitated globulins were dialysed and lyophilized. It was only after addition of a new amount of Sedormid that a positive complement test on the platelets could be obtained with the isolated globulins. The dialysis and lyophilization had withdrawn all the Sedormid which could have bound to the globulins in the serum.

More recently Horowitz (1964) observed the fixation of tritiated digitoxin to the antibody developed in a thrombocytopenic purpura induced by Digitoxin. But Horowitz observed a fixation of the tritiated drug on some normal controls and on some sera of non thrombocytopenic patients taking Digitoxin, greater than that observed on the serum of the thrombocytopenic patient.

Young, Nachman & Horowitz (1966) after incubating the serum of the Digitoxin-induced thrombocytopenic patient with tritiated Digitoxin, submitted the serum to starch gel electrophoresis. By this technique he could observe that the tritiated Digitoxin was fixed on different protein fractions of the patient's serum and of the controls, yet the fixation of the drug to the γ-globulin fraction of the patient's serum was significantly greater than that to the same protein fraction of the controls. This experiment proves, as does our own, that there is a difference between specific fixation and a non-specific fixation of the drug on the different types of protein, a specific fixation appearing on the γ-globulins of a patient sensitive to a drug, and an aspecific fixation on other protein types and on the ones of the control.

Shulman (Shulman, 1963; Shulman & Ball, 1963; Shulman 1964) studied *in vitro* the association of quinidine to the antibody of a quinidine induced thrombocytopenic patient; the purified antibody having been prepared by absorption and elution from the appropriate cells; Shulman thus measured an approximate value of the association constant of the drug and the antibody. These experiments suggest, therefore, a fairly stable association between the drug and the antibody which it had induced.

In our experiments the greater fixation of the labelled drug on the patient's serum proteins than that on the proteins of the pooled normal sera seems hardly questionable as the protein concentrations in the sera were nearly the same (60 mg/ml for the patient's serum compared to 65 mg/ml for the pooled normal sera). Furthermore, the micro-immunoelectrophoresis suggested that the IgG globulin concentration was similar to pooled normal serum but that the IgM level was slightly elevated.

Regarding the nature of the antibody, and given the different types of immunoglobulins, our results indicate the presence of the allergic antibody in the IgG (7S globulins) as well as in the IgM (19S globulins). Each of these protein groups, isolated by chromatography on DEAE-cellulose for the IgG, and by gel filtration on Sephadex G-200 for the IgM, caused leucoagglutination when in the presence of the drug (Table 9). Moreover, the treatment of the pathological serum with 2-mercaptoethanol lessened only the agglutination titre of the serum, showing that part of the antibody was certainly of the IgG type.

Regarding the fixation site of the drug, the results of our experiments suggest, as these

conclusions should be based on only one pool of normal sera, that in drug-induced allergic leucopenia, the antibody elaborated is capable of binding specifically the drug which has induced its formation. The antibody activity is found not only in the IgG (7S globulins) and in the IgM (19S globulins) but also in the IgA (9S to 10S globulins). These results have been demonstrated by showing a fixation of ^{131}I-labelled iodoantipyrine on the patient's serum; the fixation of the labelled drug was specific on to the IgM and on to the IgA globulins of the

TABLE 9. Leucoagglutination with immunoglobulin fractions

	Patient's serum		Pool of normal sera	
	Without drug	With drug	Without drug	With drug
Fractioning of the sera				
IgM	−	+	−	−
IgG	−	+	−	−

patient's serum only, as no fixation was observed on the same proteins of the pooled normal sera. On the other hand, a fixation of the labelled drug was observed on to the IgG globulins and on the proteins eluted after them, in the patient's serum as well as in the pooled normal sera, with a lesser fixation on the pooled normal sera. We must admit that the fixation of the labelled drug on to the IgG globulins of the patient's serum was only partly specific, whereas the fixation on the proteins eluted after the IgG of the patient's serum and of the pooled normal sera was nonspecific. These facts can be put together with those presented by Young *et al.* (1966) showing, by electrophoresis on starch gel, an increased fixation of tritiated Digitoxin on the γ-globulins of a Digitoxin sensitive patient's serum when compared to the fixation of the drug on to the γ-globulins of controls.

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